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(54) Title: DETECTION SYSTEM FOR HUMAN IMMUNODEFICIENCY VIRUS BASED ON NUCLEIC ACID AMPLIFICATION

(57) Abstract

Sequences allowing the detection and quantification of human immunodeficiency virus–1 (HIV–1) are described. For the detection of the various subtypes A to H (group M) sequences of a specific pol region are preferred whereby a selective group O sequence has been derived from the LTR region. Both types of sequences are either alone or in combination particularly suitable as probes in TaqMan ® analysis.

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DETECTION SYSTEM FOR HUMAN IMMUNODEFICIENCY VIRUS BASED ON NUCLEIC ACID AMPLIFICATION

Technical field

The present invention concerns oligonucleotides and oligonucleotide systems that are particularly suited for the detection and preferably also quantification of infections with human immunodeficiency viruses (HIV), in particular HIV-1.

Background Art

The rapid and specific detection of infectious agents such as HIV, in donated blood, is of utmost importance. In order to reduce the diagnostic window period, or for monitoring, sequence-based detection of viral RNA or DNA is increasingly used. So far, however, respective detection and quantification methods for HIV-1 suffer from reduced reliability due to the virus' ability for rapid mutations and existence of a broad sequence variety, as well as undesired interactions such as hairpin and duplex formation, false priming and hybridisation etc. often occurring in the samples during analysis.

It is therefore still very much desired to get tools and methods for the reliable detection and preferably quantification of HIV-1 infection independent of sequence particularities.

Disclosure of Invention

Thus, one object of the present invention was to provide sequences that enable a fast detection and quantification of HIV-1 infections by nucleic acid ampli-

fication technologies (NAT). The expression NAT comprises both sequence and signal amplification. Such methods are well known by the persons skilled in the art and comprise general PCR method, RT-PCR, Nested-PCR, antigen-capture-PCR, in-situ-PCR and TaqMan®; ligase chain reactions (LCR), in particular gap-LCR, and asymmetric gap-LCR; strand displacement amplification (SDA); and transcription-mediated amplification (TMA) or nucleic acid sequence-based amplification (NASBA); tyramide based signal amplification and branched DNA signal amplification (bDNA). The most preferred method is the so called TaqMan®-method.

It is general knowledge that primer and probe sequences are best selected in highly conserved and characteristic regions. Such regions can be found in the pol and the LTR regions of the HIV-1 genome. It has been found in connection with the present invention that the range from about nucleotide 4920 to about 5090 for pol (numbering referred to HIVHXB2CG; gene bank acc. no. K03455 and M38432, see Table 1, and SEQ. ID. NO. 1) and the range from about nucleotide 600 to about 750 for LTR (numbering referred to HIVANT70C; gene bank acc. no. L20587, see Table 2, and SEQ. ID. NO. 6) are of great importance.

Table 1:

Pol region of HIVHXB2CG (known sequence gene bank accession no. K03455 and M38432) with information on the localization of region of greatest interest(underlined) and preferred probe and primer sequences (separately marked):

```
                  4890       4900       4910       4920       4930
HIVHXB2CG   AATTCAAAATTTTCGGGTTTATTACAGGGACAGCAGAAATCCACTTTGGA
            TTAAGTTTTAAAAGCCCAAATAATGTCCCTGTCGTCTTTAGGTGAAACCT
             N  S  K  F  S  G  L  L  Q  G  Q  Q  K  S  T  L  E>
            ____POL POLYPROTEIN (NH2-TERMINUS UNCERTAIN)______>

3.  SB5s
                                                          TGGA>
                                                          ||||
HIVHXB2CG                                                 TGGA

                  4940       4950       4960       4970       4980
HIVHXB2CG   AAGGACCAGCAAAGCTCCTCTGGAAAGGTGAAGGGGCAGTAGTAATACAA
            TTCCTGGTCGTTTCGAGGAGACCTTTCCACTTCCCCGTCATCATTATGTT
              R  T  S  K  A  P  L  E  R  *  R  G  S  S  N  T>
            ____POL POLYPROTEIN (NH2-TERMINUS UNCERTAIN)______>

1.  SBP3as                                    30         20         10
                                             <AAGGTGAAGGGGCAGTAGTAATACAA
                                              ||||||||||||||||||||||||||
HIVHXB2CG                                     AAGGTGAAGGGGCAGTAGTAATACAA

3.  SB5s        10
           AAGGACCAGCAAAGC>
           |||||||||||||||
HIVHXB2CG  AAGGACCAGCAAAGC

                  4990       5000       5010       5020       5030
HIVHXB2CG   GATAATAGTGACATAAAAGTAGTGCCAAGAAGAAAAGCAAAGATCATTAG
            CTATTATCACTGTATTTTCATCACGGTTCTTCTTTTCGTTTCTAGTAATC
            R  *  *  *  H  K  S  S  A  K  K  K  S  K  D  H  *>
            ____POL POLYPROTEIN (NH2-TERMINUS UNCERTAIN)______>

1.  SBP3as
           <GATAA
            |||||
HIVHXB2CG   GATAA

2.  SB6as                          20         10
                                  <TAAAAGTAGTGCCAAGAAGAAAAG
                                   ||||||||||||||||||||||||
HIVHXB2CG                          TAAAAGTAGTGCCAAGAAGAAAAG
```

```
                5040       5050       5060       5070       5080
   HIVHXB2CG    GGATTATGGAAAACAGATGGCAGGTGATGATTGTGTGGCAAGTAGACAGG
                CCTAATACCTTTTGTCTACCGTCCACTACTAACACACCGTTCATCTGTCC
                 G  L  W  K  T  D  G  R  *  *  L  C  G  K  *  T  G>
                _____POL POLYPROTEIN (NH2-TERMINUS UNCERTAIN)_______>

                 5090       5100       5110
   HIVHXB2CG    ATGAGGATTAGAACATGGAAAAGTT
                TACTCCTAATCTTGTACCTTTTCAA
                  *  G  L>
                ____________>
```

Table 2:

LTR region of HIVANT70C (known sequence gene bank accession no. L20587) with information on the localization of region of greatest interest(underlined) and preferred probe and primer sequences (separately marked):

```
                  600        610        620        630        640
HIVANT70C         TCATCTGTTCAACCCTGGTGTCTAGAGATCCCTCAGATCACTTAGACTGA
                  AGTAGACAAGTTGGGACCACAGATCTCTAGGGAGTCTAGTGAATCTGACT
                  ____________________LTR; 5' LTR____________________>

1. SB1s                              10        20
                                     CTGGTGTCTAGAGATCCCTCAGATC>
                                     |||||||||||||||||||||||||
HIVANT70C                            CTGGTGTCTAGAGATCCCTCAGATC

                  650        660        670        680        690
HIVANT70C         AGCAGAAAATCTCTAGCAGTGGCGCCCGAACAGGGACTTGAAGTGAAAGT
                  TCGTCTTTTAGAGATCGTCACCGCGGGCTTGTCCCTGAACTTCACTTTCA
                  ___LTR; 5' LTR____>

2. SBP1s                          10        20
                                  TCTCTAGCAGTGGCGCCCGAACA>
                                  |||||||||||||||||||||||
HIVANT70C                         TCTCTAGCAGTGGCGCCCGAACA
                  700        710        720        730        740
HIVANT70C         GAAACCAGGGAAGAAAACCTCCGACGCAACGGGCTCGGCTTAGCGGAGTG
                  CTTTGGTCCCTTCTTTTGGAGGCTGCGTTGCCCGAGCCGAATCGCCTCAC

3. SB2as                                        10
                                              <GCAACGGGCTCGGCTTA
                                               |||||||||||||||||
HIVANT70C                                      GCAACGGGCTCGGCTTA

                  750
HIVANT70C         CACCCG
                  GTGGGC
```

The most interesting sequence to be used for the detection of a broad variety of HIV-1 virus subtypes (detection sequence) in the pol region comprises a sequence within or corresponding to nucleotides 4953 to 4992 with a length of 20 to 40 bp (base pairs), preferably a length of 25 to 40 bp, much preferably a length of 31 bp. The sequence chosen within said region is also referred to as hybridizing sequence or sequence with a specific hybridizing length. The whole detection sequence can be longer, i.e. it may comprise at least one non hybridizing partial sequence. The hybridizing sequence can be the specified sequence or a partial sequence within the specified range or such sequence comprising universal bases as substitute bases. At places where mismatches are expected due to nucleotide mutations, the detection sequence can comprise any replacement base which allows hydrogen-bounding with the mutated nucleotide. All these sequences are also referred to as sequences derived from the specific region. Such sequences can be obtained by generally known methods, in particular by synthesis.

The respective detection sequence thus comprises a sequence selected within or corresponding to the following sequence (SEQ. ID. NO. 2)

TATTATCTTGTATTACTACTGCCCCTTCACCTTTCCAGAG or

the respective complementary sequence, including specific G-U base pairing.

The most interesting sequence to be used for the detection of HIV-1 viruses of the O group is in the LTR region. Said sequence comprises a sequence within or is the sequence from nucleotides 653 to 680 and has a hybridizing length (see above) of 17 to 28 bp, preferably a hybridizing length of 23 bp. The respective sequence is preferably selected within the following sequence (SEQ. ID. NO. 7)

AAATCTCTAGCAGTGGCGCCCGAACAGG

whereby the sequence CGAACA is preferably present, or the

respective complementary sequence, including specific G-U base pairing.

These detection sequences can - dependent on the detection method to be used - act as primer sequences or probe sequences. In the scope of the present invention, primer and probe are distinguished in that sequences with the predominant purpose to start and maintain amplification are called primers, sequences with the predominant purpose to indicate hybridization due to specific labeling are called probes.

It has surprisingly been found that the reliability of primer and/or probe involving methods can further be enhanced if the primer(s)/probe(s), in particular the detection sequences, are selected thus that they meet or are optimized, respectively, with regard to specific requirements. Said requirements are:

- G and C content
- no duplex formation between primer(s) and/or probe(s),
- no hairpin formation within the primer(s) and/or probe(s),
- no false priming/hybridization sites for selected primer(s)/probe(s),
- no hybridization/priming with itself for the selected primer(s)/probe(s),

and, if primer(s) and as detection sequence acting probe(s) are simultaneously present,

- a T_m of the probe(s) that is about 9 to 12°C, preferably about 10 - 11 °C higher than that of the primer(s).

In the scope of the further description of the invention and the claims, wherever reference is made to sequences or sets of sequences it shall be understood

that such references concern the complementary sequences as well.

The preferred detection sequence in the pol region comprises the sequence

TTATCTTGTATTACTACTGCCCCTTCACCTT (nucleotides 4960 to 4990, SEQ. ID. NO. 3).

A preferred method for detecting and quantifying HIV-1 infection is the so called TaqMan® method largely described in the literature. Said method comprises

- a sense primer
- an antisense primer
- a probe (detection sequence, sense or antisense) comprising a labelling system allowing distinction between unbound probe and degraded probe.

A preferred labeling system is a detectable reporter covalently linked to the probe, the detectable signal of said reporter being quenched by a quencher also covalently linked to the probe, so that the detectable signal is only detected if the reporter and the quencher are separated. Such separation is obtained if a probe is first annealed to the sequence of interest and then degraded due to the PCR reaction starting from a primer also present.

It is of course also possible and it is a further object of the present invention to use different sets of sequences together, e.g. the set of the pol range and the set of the LTR range, or one or both of those in combination with at least one other set.

It is still another object of the present invention to provide a kit for such analysis such kit comprising probes and primers enabling the detection of at least one sequence of interest.

Modes for Carrying out the Invention

As already mentioned above, the present invention in one aspect concerns detection sequences, i.e. nucleotide sequences that are especially suitable to detect and quantify HIV-1 infections. Such nucleotide sequences comprise a sequence hybridizing to a particularly well conserved, characteristic region of human immunodeficiency virus-1 (HIV-1).

Dependent on the analytical method chosen, the length of the detection sequence may vary within certain limits. For the preferred, quite generally applicable pol sequence derived detection sequences, the hybridizing part should not be shorter than 20 bp and not longer than 40 bp, and it has preferably a length of about 31 bp. It is of course possible to add non-hybridizing, or non-matching, respectively, nucleotides as far as they do neither affect the desired hybridization nor the nucleic acid sequence synthesis such as PCR.

Preferred detection sequences of the present invention are uninterrupted partial sequences of the nucleotide sequence (SEQ. ID. NO. 2)

TATTATCTTGTATTACTACTGCCCCTTCACCTTTCCAGAG.

Comprising in connection with the sequences of the present invention means that they hybridize over at least the mentioned length (at most three, usually two mismatches) and may or may not have further nucleotides (non-matching or non-hybridizing, respectively) added.

Respective sequences of the LTR region are uninterrupted partial sequences of the following sequence (SEQ. ID. NO. 7)

AAATCTCTAGCAGTGGCGCCCGAACAGG

with a hybridizing length of 17 to 28 bp, preferably with a length of about 23 bp. It is furthermore preferred if the detection sequence comprises the sequence CGAACA.

Preferred detection sequences of the present invention are furthermore optimized with regard to the following characteristics:

- G and C content,
- no duplex formation between primer(s) and/or probe(s),
- no hairpin formation within the primer(s) and/or probe(s),
- no false priming/hybridization sites for selected primer(s)/probe(s),
- no priming/hybridization with itself for the selected primer(s)/probe(s),

whereby the G and C content is no criterium in the specific LTR region and would be at most about 45%, preferably less 40% in the pol region.

It has to be understood that the optimization has always to be performed for the lowest temperature reasonably applied for a specific analytical method, i.e. the most critical temperature for nucleotide interactions. Said temperature usually is about 37 to 40 °C.

Much preferred sequences being optimized with regard to said further characteristics are in the pol region

TTATCTTGTATTACTACTGCCCCTTCACCTT (SEQ. ID. NO. 3)

and in the LTR region

TCTCTAGCAGTGGCGCCCGAACA (SEQ. ID. NO. 8).

In a further aspect of the present invention, such detection sequences are not used either the one or the other, but in combination. The combined use is possible either simultaneously or one after the other. The present invention therefore also encompasses a set of detection sequences that comprises at least one of the sequences derived from the pol region and at least one of the sequences derived from the LTR region. Alternatively, it is of course also possible to use a sequence derived from the pol region and/or a sequence derived from the

LTR region in combination with other detection sequences. However, since the sequences known so far are less sensitive, such combination is not preferred at present.

In a further aspect, the present invention also provides an analytical system of primers/probe(s) comprising as at least one probe a sequence as defined above as detection sequence and having a T_m of the probe that is 9 to 12°C, preferably about 10 - 11 °C higher than the one of the primers. For such systems, the complementary strand of the detection sequence is less preferred.

A preferred analytical system or set comprises at least one analytical system of forward and reverse primer and probe selected from the group consisting of the pol region derived system.

Although the primers in such a system comprising primers and probe add to the sensitivity, selectivity and reliability of the system, they are not referred to as detection sequences within the scope of the present invention.

It has been found that preferred regions and length for primers to be used in combination with the inventive detection sequences are:

in the LTR region

primer	length in bp		range in bp
	range	optimal	
sense	19 - 33	25	600 - 650
antisense	15 - 32	17	710 - 750

in the pol region

primer	length in bp		range in bp
	range	optimal	
antisense	19 - 30	24	4995 - 5090
sense	15 - 26	19	4920 - 4955

The distance between the primer and the 5'-end of the probe for the pol system is from 1 bp to 15

bp, preferably 8 bp, in the LTR system from 1 to 20 bp, whereby 20 bp are preferred.

Much preferred analytical systems or sets of analytical systems comprise the pol region derived sequences

primer (sense) TGGAAAGGACCAGCAAAGC
(SEQ. ID. NO. 4)
primer (antisense) CCTTTCTTCTTGGCACTACTTTTA
(SEQ. ID. NO. 5)
probe (antisense) TTATCTTGTATTACTACTGCCCCTTCACCTT
(SEQ. ID. NO. 3)

and/or the LTR region derived sequences

primer (sense) CTGGTGTCTAGAGATCCCTCAGATC
(SEQ. ID. NO. 9)
primer (antisense) TAAGCCGAGCCCGTTGC
(SEQ. ID. NO. 10)
probe (sense) TCTCTAGCAGTGGCGCCCGAACA
(SEQ. ID. NO. 8).

Such analytical systems or sets of sequences or systems of course can also be present in the form of kits comprising separable specific primers and probes, enzyme reaction buffer, $MgCl_2$ stock solution, nucleotide master mix, Taq polymerase, reverse transcriptase, RNase inhibitor, positive and negative control RNA, internal run control and quantitative HIV-1 RNA standard.

The present invention also provides for a method for detecting and quantifying HIV-1. Said method comprises at least one PCR reaction involving as primer or probe one of the detection sequences described above, or a system of nucleotide sequences comprising such a detection sequence together with suitable primers. Respective methods are known to the skilled person and a selection has already been mentioned above.

A much preferred method is the so called TaqMan® method that uses a probe comprising a reporter dye and a quencher dye bound to different nucleotides of the probe such that during removal of the hybridized probe said probe is cleaved such that the reporter dye and the quencher dye are separated. Due to said separation, the reporter signal becomes detectable because of the "absence" of the quencher dye.

The TaqMan® method thus involves at least one analytical system comprising sense and antisense primers and a probe labeled with a system of reporter dye and quencher dye bound to different nucleotides of the probe. A sample to be analyzed is treated with the probe and primers under conditions first allowing hybridization of said primers and probe to a target of interest, whereupon the probe is removed due to a nucleic acid sequence synthesis starting from the corresponding primer. Due to the fact that the probe is not only separated from the target during the sequence synthesis but also cleaved, the sequence synthesis leads to a separation of the reporter dye and the quencher dye and thus to a detectable reporter signal.

A preferred TaqMan® method is based on the intrinsic 5'→ 3' exonuclease activity of preferred Taq, Tth or Tfl DNA polymerases which cleaves fluorescein (FAM; reporter dye) labelled and rhodamine (TAMRA; quencher dye) labelled probes hybridized to amplicons of the pol gene or, respectively, to the LTR gene. Probe cleavage generates specific fluorescent signals whose intensity is real-time quantified during amplification by laser-based fluorometry using a suitable detector such as e.g. the ABI PRISM 7700 Sequence Detector (PE Applied Biosystems).

In order to get a good working TaqMan® analysis, the distance between forward primer and probe has to be at least 1, usually about 4 nucleotides. The upper limit for the distance as well as the preferred distance

are dependent from the system and have already been mentioned above.

The TaqMan® method of the present invention thus provides a very suitable 5' nuclease PCR assay to rapidly detect and quantify HIV-1 RNA in plasma samples.

Preferred sequences for the TaqMan® method are

in the pol region:

primer (sense) TGGAAAGGACCAGCAAAGC
(SEQ. ID. NO. 4)

primer (anti-sense) CCTTTCTTCTTGGCACTACTTTTA
(SEQ. ID. NO. 5)

probe (anti-sense) TTATCTTGTATTACTACTGCCCCTTCACCTT
(SEQ. ID. NO. 3)

in the LTR region:

primer (sense) CTGGTGTCTAGAGATCCCTCAGATC
(SEQ. ID. NO. 9)

primer (anti-sense) TAAGCCGAGCCCGTTGC
(SEQ. ID. NO. 10)

probe (sense) TCTCTAGCAGTGGCGCCCGAACA
(SEQ. ID. NO. 8).

Using the above described TaqMan® method, with the disclosed primers and probes it was possible to reliably detect 10 copies of purified viral full-length RNA or plasmid DNA with a dynamic range of 5-6 logs of HIV-1 subtype A, B, C, D, E, F, G, H, i.e. group M, (all with the pol selective sequences) and group O (with the LTR selective sequences).

The presently described PCR system tolerates up to 3, usually 2 mismatches between the template and the 31-mer (pol) and 23-mer (LTR) probes or the flanking primers, respectively.

Best results with the above mentioned systems were found with the AMV/Tfl DNA Pol enzyme combination. Even low copy numbers of a variety of HIV-1 subtypes in

plasma specimens are quantitatively and reproducibly detectable. A TaqMan® assay of the present invention is specific and sensitive for B- and non-B HIV-1 subtypes, eliminates the need for laborious post PCR processing and permits testing of 96 samples within 2.5 hours. This makes it ideal for routine diagnostic use.

The invention is now further described by means of examples.

EXAMPLES

Example 1: PCR Primers, fluorogenic probes, target sequences and oligo design

Two pairs of probe/flanking primers in specific and highly conserved pol respective LTR regions of HIV-1 were individually designed after multiple sequence alignment of all HIV-1 subtypes (Online HIV-Sequence Database from the Los Alamos National Laboratory) using the OLIGO 5.0 primer analysis software, extended by the further criteria of the present invention.

The primer pairs and probes (Table 1) were selected under consideration of the rules that no intrinsic loop, dimer formation, internal structural instabilities (ΔG) or false priming sites should impair specific template-hybridization and of the optimal melting temperature (range 54°C-68°C).

Moreover, probe self-complementary or probe complementary to the flanking primers; 5 or more identical nucleotides, esp. Gs in a row; no G at the probes's 5'end; and a melting temperature not more than 7°C above annealing temperature, were avoided.

Because all major subtypes (and group O) of HIV-1 should be detectable, probe and primers were de-

signed to have a high cross-reactivity with all known HIV-1 strains.

Table 3:
Pol resp. LTR primers and probes

Primer or probe	Sequence (5'->3')	Fragment length (bp)
Pol: SBP3as	TTA TCT TGT ATT ACT ACT GCC CCT TCA CCT T*	31
Pol: SB5s	TGG AAA GGA CCA GCA AAG C	19
Pol: SB6as	CCT TTC TTC TTG GCA CTA CTT TTA	24
LTR: SBP1s	TCT CTA GCA GTG GCG CCC GAA CA*	23
LTR: SB1s	CTG GTG TCT AGA GAT CCC TCA GAT C	25
LTR: SB2as	TAA GCC GAG CCC GTT GC	17

*dual-labeled probe; as = antisense, s = sense

Subsequent synthesis and HPLC-grade purification of primers and fluorogenic probes (5'-labeled with reporter FAM (6-carboxyfluorescein) and 3'-labeled with quencher TAMRA (6-carboxy-tetramethyl-rhodamine) was done by PERKIN ELMER, Germany, MICROSYNTH, Switzerland and EUROGENTEC, Belgium, respectively. The TaqMan probes contained a 3'-blocking phosphate group, to prevent probe extension during PCR.

Example 2: Real-Time RT-PCR Assay and data analysis

RNA extracted from plasma, serum or cell culture supernatants was reverse transcribed and amplified in a one-tube reaction (0.05 - 0.1 ml duplicates with 0.001 - 0.025 ml diluted templates; 45 - 50 cycles) using

the Access RT-PCR System (PROMEGA) in accordance with manufacturer's instructions, slightly modified:

The 0.05 ml mixture for one PCR reaction contained, except template, 5 U of *AMV* reverse transcriptase, 5 U of *Tfl* polymerase, 4.5 mM $MgSO_4$, 0.2 mM each dNTP Mix, 400 nM of up- and downstream primers, 400-800 nM of fluorogenic probe (for primers and probes see Ex. 1), 0.007 ml optimized single-buffer 5x (Access RT-PCR System), 0.0015 ml, including the passive reference dye ROX, PCR buffer A 10x; pH 8.3 (PERKIN ELMER), 40 U recombinant RNasin Ribonuclease Inhibitor, all in nuclease-free water.

One-step reverse transcription and amplification were carried out in a single thin-wall tube in an ABI PRISM 7700 Sequence Detector based on the 5' nuclease assay. Fluorescence intensity at 518 nm is monitored continuously *real time* during DNA amplification (96-well plate format), and the data are captured onto a Macintosh computer. Run profile analysis was done by the Sequence Detection System Software (SDS Vers. 1.6.3).

The following temperature profile was used:

45 min at 48°C reverse transcription; a denaturation and AMV RT inactivation step of 2 min at 94°C; annealing at 58-62°C for 1 min with no further extension step; strand denaturation at 94°C for 15-30 s.

Because an international WHO HIV-1 Standard is still not available, two different in-house HIV-1 subtype B RNA Standards were used (38E06 - 3.8, resp. 1.5E02 - 1 copy per reaction).

Specificity of amplifications was verified by 2 % agarose gel electrophoresis and ethidium bromide staining.

Results:

Independent of virus source (plasma, serum, cell culture supernatants), it was possible to reproducibly (i.e. > 10 independent amplification experiments) detect 1-10 copies of purified HIV-1 RNA (specified subtypes A, B, C, D, E, F, G, H and O), with a dynamic range of 6 logs per 0.050 ml RT-PCR reaction.

The absolute copy numbers per reaction were determined by Amplicor testing (ROCHE Kit v.1.5). After amplification, each Taqman reaction was checked in direct comparison to a positive and a negative control on a 2% agarose gel for the prescence of the specific 91 bp-band (corresp. to the pol region) resp. the specific 127 bp-band (corresp. to the LTR region). PCR products were also confirmed by DNA sequencing. In addition, it was found that the described Taqman PCR system tolerates up to 3 mismatches between the HIV-1 template and the 31-mer pol probe resp. the flanking primers.

Example 3: Full-length HIV-1 DNA plasmids, HIV-1 PBMC gDNA, or other HIV-1 DNA preparations

Amplification of DNA templates was done under the conditions described in Example 2 without the reverse transcription step, AMV RT and RNasin.

Results:

It was possible to reliably detect purified HIV-1 PBMC-derived genomic DNA, provirus DNA and purified full-length plasmid DNA in the minimum range of 10-100 copies per 0.050 ml reaction (dynamic range of 5 logs), depending on subtype (A, B, C, E, F, H) and/or plasmid type (pBT-1, pLTR, pTZ18, pCRII, pCR2.1). Moreover, it

was also possible to detect, with the same specifity and sensitivity HIV-1 DNA from differnt preparations (e.g. Ficoll-, Trizol-, Phenol/Chloroform-, Silica-based-purified DNA).

Pol (91bp) specifity was checked together with a negative and a positive contol on a 2% agarose gel. DNA concentration per reaction was determined photometrically at 260 nm.

While there are shown and described presently preferred embodiments of the invention, it is to be distinctly understood that the invention is not limited thereto but may be otherwise variously embodied and practiced within the scope of the following claims.

Claims

1. A detection sequence comprising a nucleotide sequence hybridizing to a particularly well conserved, characteristic region of human immunodeficiency virus-1 (HIV-1), having a hybridizing length of 20 to 40 bp, said sequence being a uninterrupted partial sequence derived from the following sequence

TATTATCTTGTATTACTACTGCCCCTTCACCTTTCCAGAG

(SEQ. ID. NO. 2).

2. The nucleotide sequence of claim 1 that is optimized with regard the following characteristics:

- G and C content
- no duplex formation between primer(s) and/or probe(s),
- no hairpin formation within the primer(s) and/or probe(s),
- no false priming/hybridization sites for selected primer(s)/probe(s),
- no priming with itself.

3. The nucleotide sequence of claim 1 or 2 that comprises the sequence

TTATCTTGTATTACTACTGCCCCTTCACCTT (SEQ. ID. NO. 3).

4. The nucleotide sequence of claim 3 that is the sequence

TTATCTTGTATTACTACTGCCCCTTCACCTT (SEQ. ID. NO. 3).

5. An analytical system selected with the sequence of Table 1 (SEQ.ID.NO. 1) at least comprising a sense primer, an antisense primer and a probe (sense or antisense), wherein said probe comprises a sequence according to one of claims 1 to 4 and has a T_m of 9 to 12°C, preferably about 10 - 11 °C higher, than the one of the primers.

6. The analytical system of claim 5 wherein the sequences are selected as follows

- antisense primer with a length from 19 to 30 bp, preferably 24 bp, selected in the range from 4995 to 5090 bp,

- sense primer with a length from 15 to 26 bp, preferably 19 bp, selected in the range from 4920 to 4955 bp,

- probe with a length from 20 to 40 bp, preferably 31 bp, selected in the range from 4953 to 4992 bp,

whereby the distance between antisense primer and the 5'-end of the antisense probe is from 1 to 15 bp, preferably 8 bp.

7. The analytical system of claim 6 that comprises the following sequences

primer (sense) TGGAAAGGACCAGCAAAGC
(SEQ. ID. NO. 4)

primer (antisense) CCTTTCTTCTTGGCACTACTTTTA
(SEQ. ID. NO. 5)

probe (antisense) TTATCTTGTATTACTACTGCCCCTTCACCTT
(SEQ. ID. NO. 3)

8. A nucleotide sequence comprising as detection sequence a sequence hybridizing to a particularly well conserved, characteristic region of human immunodeficiency virus-1 (HIV-1), having a hybridizing length of 17 to 32 bp, said sequence being a uninterrupted partial sequence derived from the following sequence

AAATCTCTAGCAGTGGCGCCCGAACAGG (SEQ. ID. NO. 7), in particular a sequence comprising the partial sequence CGAACA.

9. The nucleotide sequence of claim 8 that is optimized with regard to the following characteristics:

- no duplex formation between primer(s) and/or probe(s),

- no hairpin formation within the primer(s) and/or probe(s),

- no false priming/hybridization sites for selected primer(s)/probe(s),

- no priming with itself.

10. The nucleotide sequence of claim 8 or 9 that comprises the sequence

TCTCTAGCAGTGGCGCCCGAACA (SEQ. ID. NO. 8).

11. The nucleotide sequence of claim 10 that is the sequence

TCTCTAGCAGTGGCGCCCGAACA (SEQ. ID. NO. 8).

12. An analytical system selected within the sequence of Table 2 (SEQ.ID.NO. 6) at least comprising a sense primer, a antisense primer and a probe (sense or antisense), wherein said probe comprises a sequence according to one of claims 1 to 4 and has a T_m that is about 10 - 11 °C higher than the one of the primers.

13. The analytical system of claim 12 wherein the sequences are selected as follows

- sense primer with a length from 19 to 33 bp, preferably 25 bp, selected in the range from 600 to 650 bp,
- antisense primer with a length from 15 to 32 bp, preferably 17 bp, selected in the range from 710 to 750 bp,
- probe with a length from 17 to 32 bp, preferably 23 bp, selected in the range from 651 to 690 bp,

whereby the distance between sense primer and the 5'-end of the sense probe is from 1 to 20 bp, preferably 20 bp.

14. The analytical system of claim 13 that comprises the following sequences

primer (sense) CTGGTGTCTAGAGATCCCTCAGATC
(SEQ. ID. NO. 9)

primer (anti-sense) TAAGCCGAGCCCGTTGC
(SEQ. ID. NO. 10)

probe (sense) TCTCTAGCAGTGGCGCCCGAACA
(SEQ. ID. NO. 8)

15. A set of nucleotide sequences that comprises at least one of the sequences of any one of claims

1 to 7 and at least one of the sequences of any one of claims 8 to 14.

16. The set of claim 15 that comprises the following sequences

primer (sense) TGGAAAGGACCAGCAAAGC
(SEQ. ID. NO. 4)
primer (antisense) CCTTTCTTCTTGGCACTACTTTTA
(SEQ. ID. NO. 5)
probe (antisense) TTATCTTGTATTACTACTGCCCCTTCACCTT
(SEQ. ID. NO. 3)
and
primer (sense) CTGGTGTCTAGAGATCCCTCAGATC
(SEQ. ID. NO. 9)
primer (antisense) TAAGCCGAGCCCGTTGC
(SEQ. ID. NO. 10)
probe (sense) TCTCTAGCAGTGGCGCCCGAACA
(SEQ. ID. NO. 8).

17. A method for detecting and quantifying HIV-1 in a sample by nucleic acid amplification technology that comprises at least one detection sequence selected from the sequences of any one of claims 1 to 4 or at least one detection sequence selected from the sequences of any one of claims 8 to 11 or at least one set of claim 15.

18. A method for detecting and quantifying HIV-1 in a sample by nucleic acid amplification technology that comprises at least one analytical system of one of claims 5 to 7 or at least one analytical system of one of claims 12 to 14 or at least one set of claim 16.

19. The method of claim 18 wherein the probe comprises a reporter dye and a quencher dye bound to different nucleotides of the probe, and wherein the sample is treated with the probe and primers under conditions first allowing hybridization of the probe and the primers with a target of interest followed by removal of the probe due to nucleic acid sequence synthesis, whereby the removal of the probe leads to a cleavage of the probe

thus that the reporter dye and the quencher dye are separated.

20. The method of claim 19 wherein the nucleic acid sequence synthesis is a polymerase chain reaction.

SEQUENCE LISTING

<110> Nationales Zentrum für Retroviren
Bosbach, Stefan
Böni, Jürg
Schüpbach, Jörg

<120> Detectin system for human immunodeficiency virus

<130> Nationales Zentrum für Retroviren

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INTERNATIONAL SEARCH REPORT

In. ational Application No
PCT/IB 99/00828

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/70 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	EP 0 403 333 A (INST NAT SANTE RECH MED ;PASTEUR INSTITUT (FR)) 19 December 1990 (1990-12-19) 93.3% identity in 16 bp overlap between Seq ID No 3 of application and sequence MMy32bis, page 20. page 15, line 17 - line 31; claim 4 --- -/--	1-7, 15-20

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 January 2000	04/02/2000

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INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/IB 99/00828

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 727 497 A (HOFFMANN LA ROCHE) 21 August 1996 (1996-08-21) 100% identity in 12 bp overlap between Seq ID No 6 and that of Seq ID No 4 of present application. claims 1-15; example 6	1-7, 15-20
X	US 5 478 724 A (MORSE STEPHEN S ET AL) 26 December 1995 (1995-12-26) the whole document	1-7, 15-20
X	EMBL Database Entry X15222 Accession No: X15222; 29-April 1999 Progenics Pharm Inc. " HUMAN IMMUNODEFICIENCY VIRUS TYPE 1" XP002128584 100% identity in 28 bp overlap between seq X15222 and that of Seq ID No 7 of present application	8-11

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INTERNATIONAL SEARCH REPORT

Information on patent family members

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PCT/IB 99/00828

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